

Na⁺/K⁺ ATPase α 1 and β 3 subunits are localized to the basolateral membrane of trophoctoderm cells in human blastocysts

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STUDY QUESTION: Is there a relation between specific Na⁺/K⁺ ATPase isoform expression and localization in human blastocysts and the developmental behavior of the embryo?

SUMMARY ANSWER: Na⁺/K⁺ ATPase α 1, β 1 and β 3 are the main isoforms expressed in human blastocysts and no association was found between the expression level of their respective mRNAs and the rate of blastocyst expansion.

WHAT IS KNOWN ALREADY: In mouse embryos, Na⁺/K⁺ ATPase α 1 and β 1 are expressed in the basolateral membrane of trophoctoderm (TE) cells and are believed to be involved in blastocoel formation (cavitation).

STUDY DESIGN, SIZE, DURATION: A total of 20 surplus embryos from 11 patients who underwent IVF and embryo transfer at a university hospital between 2009 and 2018 were analyzed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After freezing and thawing Day 5 human blastocysts, their developmental behavior was observed for 24h using time-lapse imaging, and the expression of Na⁺/K⁺ ATPase isoforms was examined using quantitative RT-PCR (RT-qPCR). The expressed isoforms were then localized in blastocysts using fluorescent immunostaining.

MAIN RESULTS AND THE ROLE OF CHANCE: RT-qPCR results demonstrated the expression of Na⁺/K⁺ ATPase α 1, β 1 and β 3 isoforms in human blastocysts. Isoforms α 1 and β 3 were localized to the basolateral membrane of TE cells, and β 1 was localized between TE cells. A high level of β 3 mRNA expression correlated with easier hatching ($P=0.0261$).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The expression of mRNA and the localization of proteins of interest were verified, but we have not been able to perform functional analysis.

WIDER IMPLICATIONS OF THE FINDINGS: Of the various Na⁺/K⁺ ATPase isoforms, expression levels of the α 1, β 1 and β 3 mRNAs were clearly higher than other isoforms in human blastocysts. Since α 1 and β 3 were localized to the basolateral membrane via fluorescent immunostaining, we believe that these subunits contribute to the dilation of the blastocoel. The β 1 isoform is localized between TE cells and may be involved in tight junction formation, as previously reported in mouse embryos.

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Key words: human blastocyst / blastocoel / Na⁺/K⁺ ATPase / subunit / isoform / time-lapse imaging / immunostaining

Introduction

ESHRE has reported that the introduction of time-lapse imaging has enabled more extensive observations and reports on the developmental behaviors of blastocysts (ESHRE Working group on Time-lapse technology, 2020). A better understanding of some of these behaviors, including blastocoel formation and blastocyst expansion, may potentially lead to the selection of good quality human embryos. For example, Huang et al. (2019) reported that the rate of expansion of euploid human blastocysts is significantly faster than that of aneuploid blastocysts. Bell et al. (2008) concluded that the following two mechanisms underlie blastocoel formation (cavitation) in mammalian animals: expression of sealing function by the formation of tight junctions between trophoblast cells; and influx of water into the blastocoel through the aquaporin channel. This occurs according to the ion concentration gradient created by the uptake of Na⁺ into the blastocoel by the Na⁺/K⁺ ATPase present in the basolateral membrane of trophectoderm (TE) cells. In addition, Madan et al. (2007) reported that Na⁺/K⁺ ATPase β 1 subunit may be involved in tight junction formation in a Na⁺/K⁺ ATPase β 1 isoform-knockout animal model.

Na⁺/K⁺ ATPase is composed of three subunits, α , β and γ . The α subunit, which is the catalytic unit, has four isoforms (α 1, α 2, α 3, α 4), the β subunit has three isoforms (β 1, β 2, β 3) and the γ subunit has seven isoforms (FXDY1-7) (Felippe Gonçalves-de-Albuquerque et al., 2017). Each human organ has specific Na⁺/K⁺ ATPases and the expression patterns of the different isoforms vary. For human embryos, the β 3 isoform has been shown to be strongly expressed at the mRNA level in TE cells (Adjaye et al., 2005); however, other isoforms have not been investigated.

An association between the rate of blastocyst expansion and pregnancy rate has been reported (Shu et al., 2009; Yin et al., 2016; Kovačić et al., 2018). Hence, clarifying the expansion mechanism of blastocysts is expected to lead to new insights into embryo evaluation and the selection of good quality embryos. Rather than relying on the animal experimental models detailed in previous reports (Bell et al., 2008), it is necessary to identify which isoforms of the Na⁺/K⁺ ATPase subunits that are believed to be involved in blastocoel expansion contribute to the unique developmental behavior of the human embryo.

In this study, we aimed to clarify the expression and localization of Na⁺/K⁺ ATPase isoforms in human blastocysts and to investigate their relation to embryonic developmental behavior. Frozen-thawed day 5 (D5) human blastocysts were observed using time-lapse imaging for 24h, and the expression of each isoform was quantified by quantitative RT-PCR (RT-qPCR). Furthermore, the localization of the expressed isoforms in the blastocyst was examined by fluorescent immunostaining.

Materials and methods

Patients and embryos

In this study, 20 surplus embryos were used after obtaining informed consent in writing from 11 patients who were treated at Akita University Hospital, Akita, Japan, between 2009 and 2018. The average age of patients at embryo freezing was 32.64 ± 3.23 years

(Table I). All surplus embryos used were fertilized by IVF and frozen on D5. Embryo grades were evaluated using the Gardner classification (Gardner and Schoolcraft, 1999) at the time of freezing, and 3BB or higher was regarded as a good quality embryo while grades below this indicated poor quality embryos (Table II).

Table I Characteristics of patients who provided the frozen surplus embryos.

Characteristics	N = 11
Age (mean \pm SD, years)	32.64 \pm 3.23
BMI (mean \pm SD, kg/m ²)	21.85 \pm 2.39
Diagnosis of infertility	
Tubal	2
Endometriosis	2
PCOS	3
Unexplained	3
For fertilization	1

PCOS, polycystic ovary syndrome.

Table II Characteristics of frozen surplus embryos and the presence or absence of hatching after thawing.

No.	Grade*	dpf	Fertilization method	Patient no.	Hatching
1	3BA	5	IVF	1	Hatched
2	3CB	5	IVF	1	Hatched
3	4AA	5	IVF	2	Hatched
4	3BB	5	IVF	3	Hatched
5	3CC	5	IVF	3	Non-hatched
6	3AB	5	IVF	4	Non-hatched
7	3BC	5	IVF	4	Non-hatched
8	4BC	5	IVF	5	Non-hatched
9	4AA	5	IVF	6	Non-hatched
10	4AB	5	IVF	7	Hatched
11	4CC	5	IVF	7	Non-hatched
12	4AB	5	IVF	8	Hatched
13	4BA	5	IVF	8	Hatched
14	4AA	5	IVF	9	Non-hatched
15	4AA	5	IVF	10	Non-hatched
16	3AB	5	IVF	4	Hatched
17	3BC	5	IVF	4	Non-hatched
18	4AA	5	IVF	11	Non-hatched
19	3BB	5	IVF	11	Non-hatched
20	3BB	5	IVF	11	Non-hatched

dpf, days post-fertilization.

*Embryo grades were evaluated using the Gardner classification (Gardner and Schoolcraft, 1999) at the time of freezing on Day 5, and 3BB or higher was regarded as a good quality.

Embryo thawing, culture and time-lapse imaging

Embryos were thawed using the Cryotop Safety Kit (Kitazato, Japan) according to the manufacturer's protocol. Subsequently, embryos were cultured in Sequential Blast™ medium (ORIGIO, Denmark) in a time-lapse incubator (Primo Vision; Vitrolife, Sweden) at 37°C, with 5% O₂, 5% CO₂ and 90% N₂, for 24 h. Time-lapse images were taken every 5 min and blastocyst diameters were measured from still images every 20 min. The starting point was taken as the point where the diameter of the embryo was smallest after the start of observation, and the endpoint was taken as the point immediately before the diameter was reduced by contraction. Various points between the starting and endpoints were plotted at regular intervals, and the slope of the fitted line was defined as the expansion rate (μm/min) (Fig. 1).

RT-qPCR

Prior to RNA extraction, embryos were briefly exposed to acidic Tyrode's solution (Kitazato, Japan) to remove the zona pellucida. Total RNA was extracted using a RNeasy Micro Kit (Qiagen, Hilden, Germany) with DNase treatment, according to the manufacturer's protocol. RNA quality and quantity were measured using a NanoDrop™ (Thermo Fisher Scientific, Wilmington, DE, USA) device. RNA was reverse transcribed into cDNA using the PrimeScript IV 1st strand cDNA Synthesis Mix (TAKARABIO, Japan) with RNase

inhibitor, according to the manufacturer's protocol. RNA was then cryopreserved at -20°C until use.

Since the amount of mRNA contained in one embryo is small, the cDNA was amplified as follows: TaqMan (20×) gene expression assays of seven Na⁺/K⁺ ATPase isoform target genes and five reference genes (Table III) were combined. A primer pool was prepared so that the final concentration of each assay was 0.2× using 1× TE buffer. Using TaqMan PreAmp Master Mix (Thermo Fisher Scientific; # 4391128), preamplification was performed in 14 cycles according to the manufacturer's protocol.

A LightCycler 480 system II (Roche, Basel, Switzerland) was used for RT-qPCR. Preamplification products, diluted 1:20 with 1× TE buffer, were amplified using 20× TaqMan gene expression assays and TaqMan Gene Expression Master Mix, according to the manufacturer's procedure (Thermo Fisher Scientific; #4369016). A standard RT-qPCR protocol was used, consisting of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Three technical replicates were assayed for each biological sample, and the average cycle threshold (Ct) value of the triplicate samples was calculated. A no-template control was also included to check whether primer-dimers or contamination with amplified PCR product was detectable. The gene expression level was determined using the $\Delta\Delta C_t$ method (Silver *et al.*, 2006) and Ref Finder (<http://150.216.5.6.64/referencegene.php>) was used for normalization of Ct values.

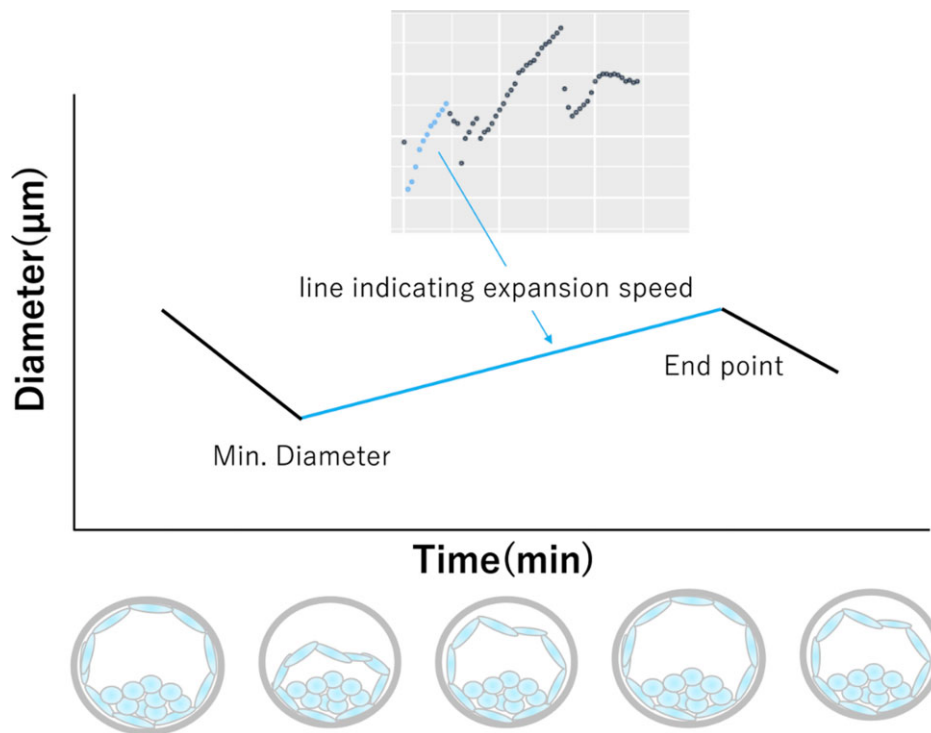


Figure 1. Definition and measurement of human blastocyst expansion rate. From among the various blastocyst diameter measurements collected by time-lapse imaging, the starting point was set as the point when the diameter of the embryo was the smallest after the start of observation. The endpoint was set as the point immediately prior to the beginning of contraction, when the diameter of the embryo decreased. Data between these two points were plotted, and the slope of the fitted line was defined as the expansion rate (μm/min).

Table III Primers of seven Na⁺/K⁺ ATPase isoform target genes and five reference genes.

Target	Full name	Taqman probe assay ID	Dye
ATPIA1	ATPase Na ⁺ /K ⁺ transporting subunit alpha 1	Hs00167556_m1	FAM
ATPIA2	ATPase Na ⁺ /K ⁺ transporting subunit alpha 2	Hs00265131_m1	FAM
ATPIA3	ATPase Na ⁺ /K ⁺ transporting subunit alpha 3	Hs00958036_m1	FAM
ATPIA4	ATPase Na ⁺ /K ⁺ transporting subunit alpha 4	Hs00380134_m1	FAM
ATPIB1	ATPase Na ⁺ /K ⁺ transporting subunit beta 1	Hs00426868_g1	FAM
ATPIB2	ATPase Na ⁺ /K ⁺ transporting subunit beta 2	Hs01020302_g1	FAM
ATPIB3	ATPase Na ⁺ /K ⁺ transporting subunit beta 3	Hs00740857_mH	FAM
PRLP0	Ribosomal protein lateral stalk subunit P0	Hs99999902_m1	FAM
ACTB	Actin beta	Hs99999903_m1	FAM
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	FAM
RAF1	Raf-1 proto-oncogene, serine/threonine kinase	Hs00234119_m1	FAM
CTNNB1	Catenin beta-1	Hs00355045_m1	FAM

Immunofluorescence

To clarify the localization of the Na⁺/K⁺ ATPase isoforms $\alpha 1$, $\beta 1$ and $\beta 3$, immunofluorescent staining and analysis were performed in accordance with our previously published methods on mouse embryos (Fujishima et al., 2021). The following mouse monoclonal IgG antibodies were used: ATPIA1(C464.6) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-21712; 200-fold dilution), ATPIB1(C464.8) (Santa Cruz Biotechnology; sc-21713; 200-fold dilution), and ATPIB3(46) (Santa Cruz Biotechnology; sc-135998; 50-fold dilution).

Statistical analyses

All statistical analyses were performed using The R Project for Statistical Computing (R Version 4.0.2, Vienna, Austria). The Wilcoxon rank sum test or the paired Student's *t*-test was used to compare the expansion rates and mRNA expression levels between groups. The relation between the expansion rate and mRNA expression level was determined using Pearson's correlation coefficient. A value of $P < 0.05$ was considered to indicate statistical significance.

Ethical approval

This original research study was approved by the Ethical Committee of Akita University (Permission number: 1090). The methods were carried out in accordance with the relevant regulations on research of human sperm/ovum/fertilized eggs set forth by the Japan Society of Obstetrics and Gynecology.

Results

Expression levels of Na⁺/K⁺ ATPase isoforms mRNA

The distribution of average Ct values for the expression of Na⁺/K⁺ ATPase isoforms showed that $\alpha 1$, $\beta 1$, and $\beta 3$ were the main isoforms expressed in the 20 human blastocysts studied (Fig. 2).

Localization of $\alpha 1$, $\beta 1$ and $\beta 3$

The Na⁺/K⁺ ATPase isoform $\alpha 1$, $\beta 1$ and $\beta 3$ mRNAs were expressed in human blastocysts. Analysis by fluorescent immunostaining revealed that $\alpha 1$ and $\beta 3$ proteins were localized to the basolateral membrane of TE cells, and $\beta 1$ was localized between TE cells (Fig. 3).

Time course of blastocyst diameter measurements

Changes in diameter of each of the 20 embryos were measured up to hatching or 24 h post-thawing (Fig. 4). Most embryos exhibited weak contractions or collapsed after thawing. To eliminate the influence of contraction on our data, the expansion rate was calculated using data measured between the previously described starting (diameter of the embryo was smallest after thawing) and endpoints (immediately before the diameter was reduced by contraction) (Figs 1 and 4, light blue dots).

Relation between expansion rate and Na⁺/K⁺ ATPase mRNA expression level

No correlation was found between the mRNA expression levels of $\alpha 1$, $\beta 1$ and $\beta 3$ and the expansion rate (Fig. 5). No significant difference in the expansion rate was observed following comparisons of embryos divided into hatch ($n = 8$) and non-hatch ($n = 12$) groups, and good quality embryo ($n = 14$) and poor quality embryo ($n = 6$) groups (Fig. 6A and B). However, when mRNA expression levels were compared, the $\beta 3$ mRNA expression level was found to be significantly higher in the hatch group than that in the non-hatch group ($P = 0.0261$). There was no significant difference between the groups for $\alpha 1$ and $\beta 1$ mRNA expression (Fig. 6C and D).

Discussion

In this study, the expression and localization of Na⁺/K⁺ ATPase isoforms were examined in human blastocysts. We showed that $\alpha 1$, $\beta 1$

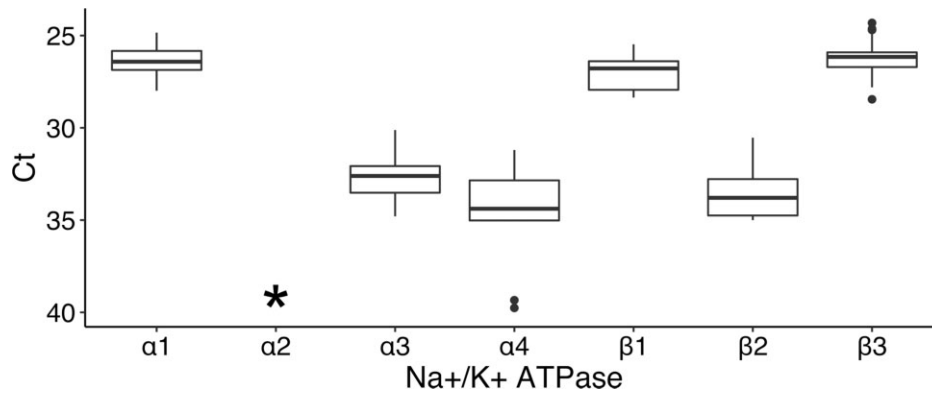


Figure 2. Expression of Na⁺/K⁺ ATPase isoforms in human blastocysts based on quantitative RT-PCR cycle threshold values. Boxplots for average Ct of three technical replicates for the seven Na⁺/K⁺ ATPase isoforms. The box shows the 25/75 percentile. A line across the box indicates the median. The main isoforms in the 20 human blastocysts were α1, β1 and β3. *α2 was not detected by 40 cycles of PCR.

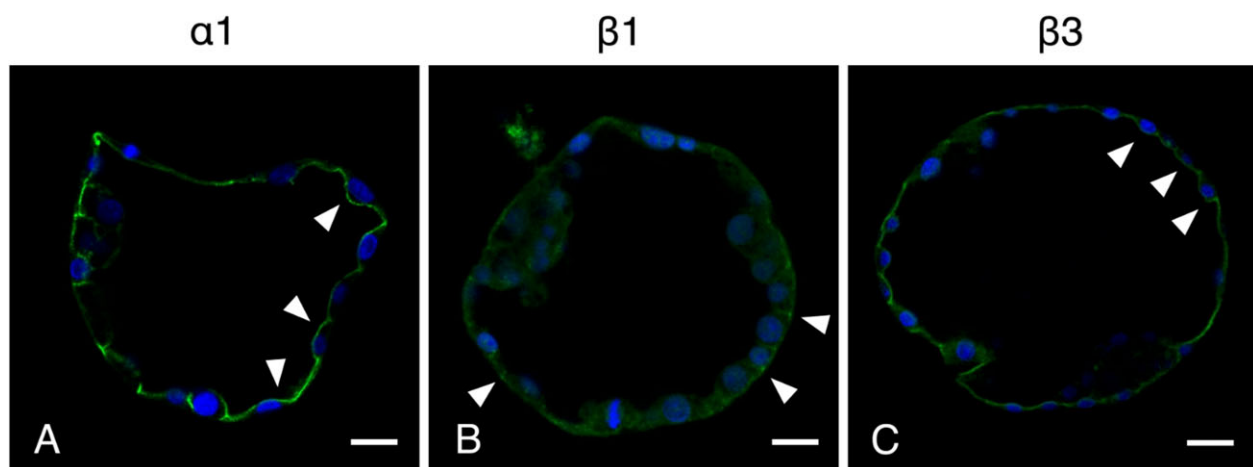


Figure 3. Immunofluorescent localization of Na⁺/K⁺ ATPase isoforms in human blastocyst. (A, C) α1 and β3 were localized to the basolateral membrane of trophoblast (TE) cells (arrowheads), and (B) β1 was localized between TE cells (arrowheads). Scale bar, 30 μm.

and β3 were expressed in human blastocysts and that α1 and β3 were localized to the basolateral membrane of TE cells, while β1 was localized between TE cells. In mouse embryos, α1 and β1 are located in the basolateral membrane of TE cells, and they are considered to be involved in the expansion of the blastocoel (Bell *et al.*, 2008); in human embryos, α1 and β3 are thought to play the same role.

We assumed that two factors are involved in regulating the Na⁺ concentration in the blastocoel: the expression level of Na⁺/K⁺ ATPase and the functional activity of Na⁺/K⁺ ATPase. There are no reports of direct quantitative observation of electrolyte behavior during human embryo development; therefore, we focused on the expansion rate of blastocysts as a method for evaluating the activity of Na⁺/K⁺ ATPase. Higher activity of Na⁺/K⁺ ATPase is expected to be related to a faster expansion rate. However, in this study, no correlation was

found between the α1 or β3 mRNA expression level and the expansion rate; thus, the expansion rate may not be determined by the activity of Na⁺/K⁺ ATPase alone. As mentioned earlier, the strength of the tight junction may also be relevant. Further, the expansion rate may depend on the number of aquaporin channels that are the inflow path for water.

The β3 mRNA expression level was significantly higher in the hatched group than that in the non-hatched group. We believe that this result supports the high ion-exchange activity facilitated by Na⁺/K⁺ ATPase in the hatched group. However, the β subunit of Na⁺/K⁺ ATPase exists in the membrane protein as a scaffold, while the α subunit is bound to this scaffold and is the catalyst unit that actually performs the ion exchange. Thus, if the higher the Na⁺/K⁺ ATPase activity the easier it is to hatch, there may also be a relation between

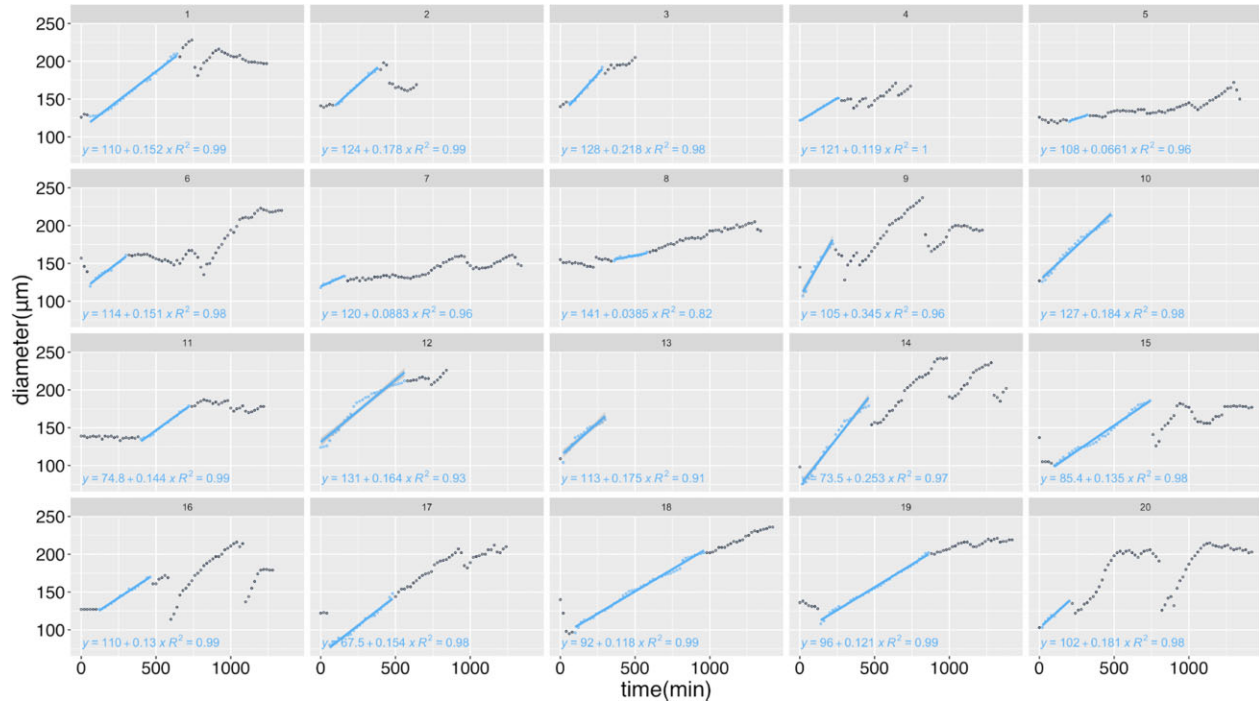


Figure 4. Diameter of human blastocysts. The diameter of the blastocysts ($n = 20$) was measured up to hatching or 24 h post-thawing. The slope of the line fitted to the plotted data (light blue), from the minimum diameter immediately after thawing to the diameter before contraction, was used as the expansion rate.

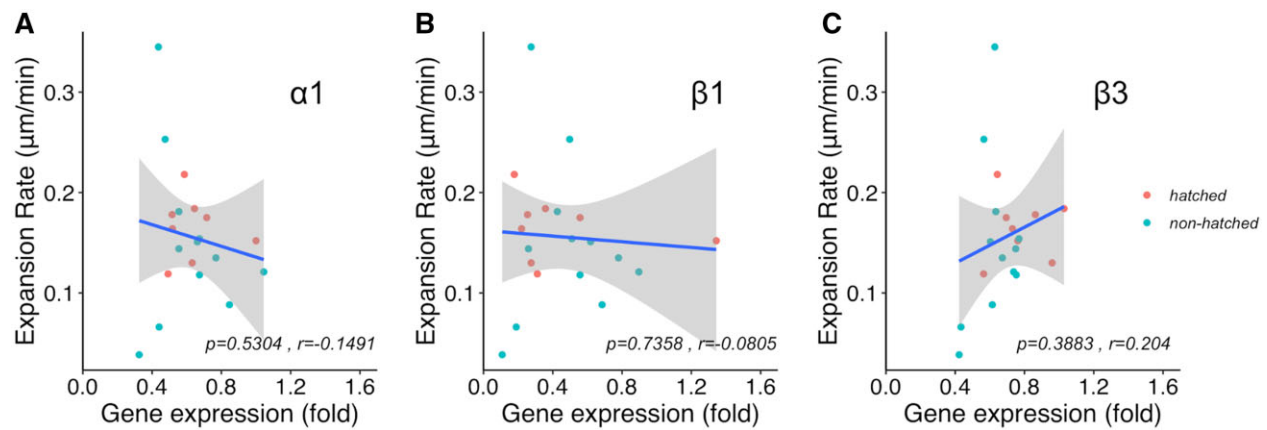


Figure 5. Correlation between mRNA expression levels of Na⁺/K⁺ ATPase isoforms and the expansion rate of human blastocysts. The mRNA expression levels of Na⁺/K⁺ ATPase (A) α 1, (B) β 1 and (C) β 3 and the expansion rate. No correlation was detected between these levels and expansion rate.

α 1 mRNA expression level and hatching, but this was not the case in this study. Additionally, it may be necessary to look at protein expression as well. From another perspective, it can be considered that the β 3 mRNA expression level increases after hatching, not before. In this case, β 3 may not act as a regulatory unit for Na⁺/K⁺ ATPase but

may instead play a different role in embryonic development toward implantation after hatching. Bovine embryos that are fertilized *in vitro* have a higher blastocoel expansion rate than that of *in vivo* fertilized bovine embryos; however, the expression level of β 3 mRNA is also high in *in vitro* fertilized bovine embryos (Goossens et al., 2007).

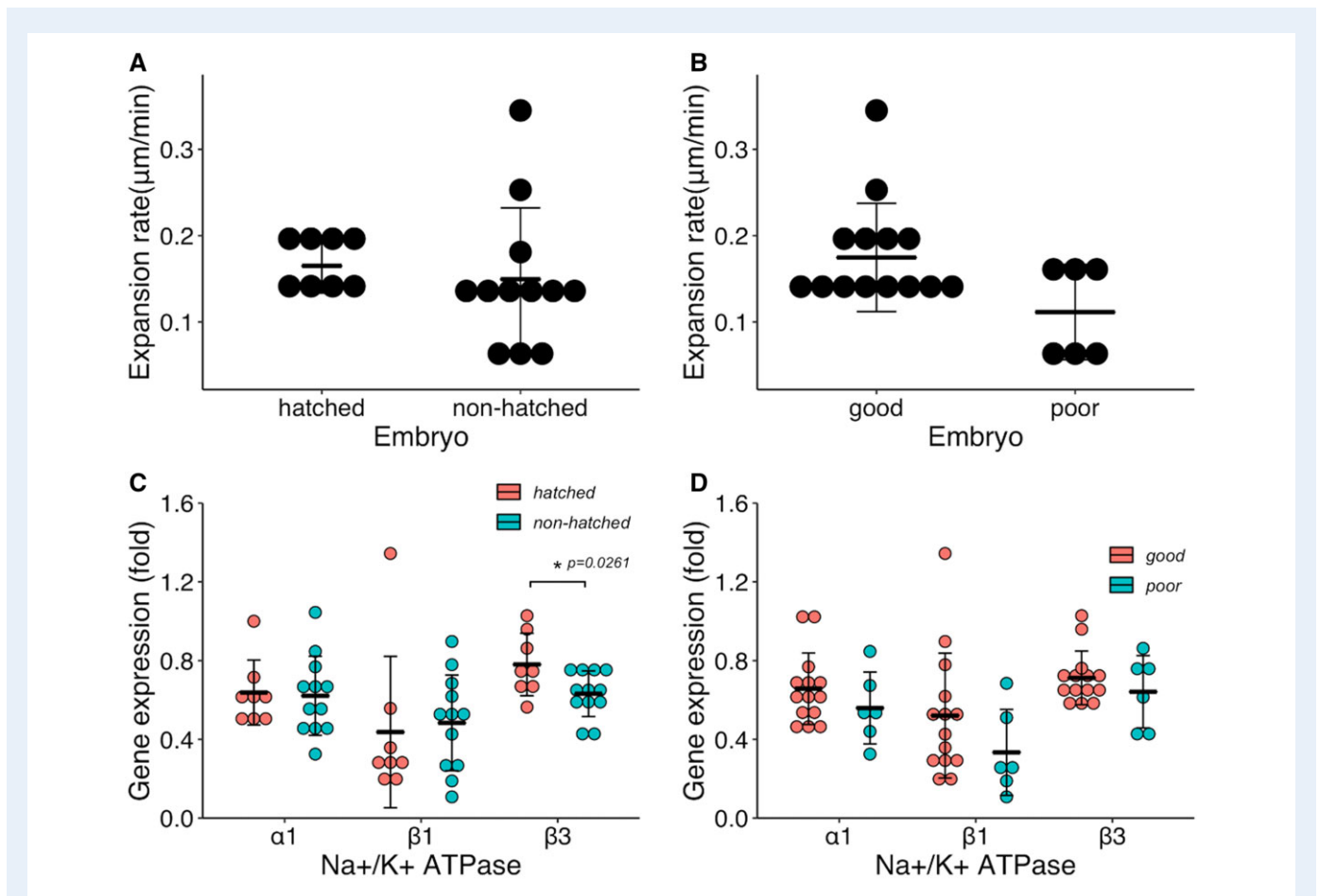


Figure 6. Gene expression of Na⁺/K⁺ ATPase isoforms and expansion rate of human embryos (n = 20). Expansion rate in (A) hatched versus non-hatched groups ($P = 0.563$, Student's t -test) and (B) good versus poor quality groups ($P = 0.2$, Wilcoxon rank sum test). mRNA expression of isoforms in (C) hatched ($n = 8$, red) versus non-hatched ($n = 12$, green) groups ($\alpha 1$, $P = 0.895$, Wilcoxon rank sum test; $\beta 1$, $P = 0.373$, Wilcoxon rank sum test; $\beta 3$, $P = 0.0261$, Student's t -test) and (D) good ($n = 14$, red) versus poor ($n = 6$, green) quality groups ($\alpha 1$, $P = 0.284$, Student's t -test; $\beta 1$, $P = 0.146$, Wilcoxon rank sum test; $\beta 3$, $P = 0.718$, Wilcoxon rank sum test).

The expression of $\beta 3$ subunit is also higher in gastric cancer than that in healthy matched tissues. $\beta 3$ is involved in cell proliferation through the PI3K/AKT (phosphatidylinositol 3-kinase/protein kinase B) signaling pathway, and cells in which $\beta 3$ is knocked down tend to undergo apoptosis (Li *et al.*, 2017). Similar reports on hepatocellular carcinoma suggest that $\beta 3$ may be a biomarker for prognostic prediction (Lu *et al.*, 2021). These reports suggest that $\beta 3$ may play a role during the adaptation of cells to environments that differ from the *in vivo* environment or that require higher energy efficiency. The embryos examined in the present study were surplus embryos from IVF procedures that had been subjected to freeze-thawing. The possibility that they underwent significant stress during their development processes cannot be denied.

The $\beta 1$ subunit is involved in the normal distribution of Na⁺/K⁺ ATPase and the localization of proteins that make up tight junctions in mouse embryos (Madan *et al.*, 2007). In human alveolar epithelial cells, tight junctions are also regulated by myotonic dystrophy kinase-related cdc42-binding kinase (MRCK α) (Bai *et al.*, 2021). In our study, immunostaining revealed $\beta 1$ expression between the TE cells of human

embryos, suggesting that $\beta 1$ is also involved in tight junction formation in human embryos.

The expansion phenomenon of mammalian blastocysts has been studied relative to the dynamics of Na⁺ and K⁺ and their related molecules. In particular, the dynamics of Na⁺ and K⁺ migration in blastocyst dilation have been shown, albeit indirectly (Bell *et al.*, 2008); they hypothesized that blastocysts expand as water flows into the blastocoel with the Na⁺ concentration gradient created by the Na⁺/K⁺ ATPase present in the TE cell. More recently, we directly observed intracellular and blastocoel electrolyte concentrations in the mouse blastocyst using electrolyte indicator (Fujishima *et al.*, 2021). Then, we confirmed the dynamics of Na⁺ and K⁺ migration, which are consistent with the above hypothesis. However, no quantitative measurements were made. It is the active Na⁺/K⁺ ATPase protein on the basolateral membrane side that is involved in ion exchange (Yoshimura *et al.*, 2008). Therefore, as described above, it seems that the expression level of Na⁺/K⁺ ATPase protein and/or its substantial activity is involved in blastocyst expansion. In terms of activity, the Na⁺/K⁺ ATPase transports three Na⁺ to the outside of the cell,

consuming one molecule of ATP in taking up two K⁺ into the cell. However, consumption of ATP is a general intracellular biological activity, making detection of individual reactions or sites of ATP consumption very difficult. If a detection system could be designed to quantitatively measure ATP associated with a particular reaction or site, it is possible that monitoring these specific changes in ATP could contribute to embryo selection. It should also be noted that the function of Na⁺/K⁺ ATPase isoforms differs depending on the animal species. Further verification of the role of the Na⁺/K⁺ ATPase in development and the homology of its subunit isoforms are needed.

In conclusion, we found that expression levels of the $\alpha 1$, $\beta 1$ and $\beta 3$ mRNAs were clearly higher than other isoforms in human blastocysts. Subunits $\alpha 1$ and $\beta 3$ were localized to the basolateral membrane and $\beta 1$ was localized between cells. No correlation was found between the $\alpha 1$ or $\beta 3$ mRNA expression level and the expansion rate, but the $\beta 3$ mRNA expression level was significantly higher in the hatched group than that in the non-hatched group.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

T.H., K.T. and Y.T. designed the study. T.H., M.G. and K.T. performed experiments. T.H., A.F., K.M. and T.S. performed data analysis and interpretation. T.H. wrote the manuscript. K.T., K.M., H.S., W.S., Y.K. and Y.T. reviewed the manuscript.

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Conflict of interest

None declared.

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